

APPLICATION
FOR
UNITED STATES LETTERS PATENT

TITLE: AUTOANTIGEN VACCINE

APPLICANT: TIHAMER ORBAN

CERTIFICATE OF MAILING BY EXPRESS MAIL

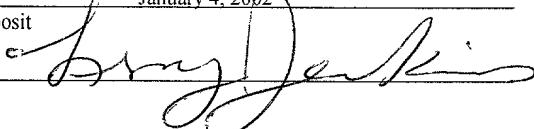
Express Mail Label No EL 486 014 455 US

I hereby certify under 37 CFR §1.10 that this correspondence is being deposited with the United States Postal Service as Express Mail Post Office to Addressee with sufficient postage on the date indicated below and is addressed to the Commissioner for Patents, Washington, D.C 20231.

Date of Deposit

January 4, 2002

Signature



Leroy Jenkins

Typed or Printed Name of Person Signing Certificate

AUTOANTIGEN COMPOSITION

RELATED APPLICATIONS

This application claims the benefit of U.S. Provisional Application Serial No. 60/260,068, filed January 5, 2000, the contents of which are incorporated herein by reference 5 in their entirety.

BACKGROUND

Despite the significant progress that has been made in its treatment, diabetes represents a severe burden on the individual as well as society. Insulin dependent diabetes mellitus (Type 1 diabetes) is an autoimmune disease, where insulitis leads to the destruction 10 of pancreatic β -cells. At the time of clinical onset of type 1 diabetes mellitus, significant number of insulin producing β cells are destroyed and only 15% to 40% are still capable of insulin production (McCulloch et al. (1991) *Diabetes* 40:673-679). β -cell failure results in a life long dependence on daily insulin injections and exposure to the acute and late 15 complication of the disease. The natural history of the disease is that the remaining functional population of β -cells inevitably dies, rendering the patients dependent on exogenous insulin for life. Arrest or even the slow down of further destruction of β -cells 20 would lead to prolonged remission period and delay the diabetes-related complications.

Insulin, which is a β -cell specific major protein and is also moderately immunogenic when used alone, has been shown in a pilot human trial to have the effect of delaying the development of diabetes mellitus (Keller et al. (1993) *Lancet* 341:927-928). However, it must be injected daily over long periods of time to induce the desired effect. Also, the use of 25 insulin implicates a concern about hypoglycemia and its sequels.

Reintroduction of autoantigen, such as insulin B chain, in incomplete Freund's adjuvant (IFA), has been used in animal models of diabetes, such as NOD mice (Muir et al. 25 (1995) *J Clin Invest* 95:628-634; Orban et al. (1999) *Diabetes* 48 Supp.1:A216-A217; Ramiya et al. (1996) *J Autoimmun* 9:349-356).

SUMMARY OF THE INVENTION

In one aspect, the invention features a method for the prevention or treatment of an autoimmune disorder, e.g., rheumatoid arthritis, multiple sclerosis, or diabetes, e.g., type 1 diabetes. The methods include administering to a subject, e.g., a human, preferably a non-immunocompromised or non-immunosuppressed human, a composition, e.g., a vaccine, which contains an autoantigen and an oil-based carrier, e.g., an oil-based adjuvant, e.g., incomplete Freund's adjuvant (IFA), Montanide ISA (e.g., Montanide ISA51) or an equivalent composition. In preferred embodiments, the oil-based carrier or adjuvant, and preferably the composition, does not include a bacterial component, e.g., a mycobacterial component.

10 In a preferred embodiment, the subject is at risk for type 1 diabetes.

15 In another preferred embodiment, the subject exhibits autoimmunity, e.g., against a diabetes autoantigen.

20 In a preferred embodiment, the autoantigen is a diabetes type 1 autoantigen, e.g., an islet cell autoantigen.

25 In a preferred embodiment, the autoantigen is preproinsulin or an immunologically active fragment or variant thereof, e.g., human insulin A-chain, B-chain, C peptide or an immunologically active fragment or variant thereof, e.g., the autoantigen is a fragment of the sequence shown as SEQ ID NO:1.

30 In a preferred embodiment, the autoantigen is human insulin B-chain (amino acids 25-54 of SEQ ID NO:1) or an immunologically active fragment, or variant thereof.

35 In a preferred embodiment, the B-chain or fragment thereof is not recombinant. For example, the B-chain or immunogenic fragment or variant thereof is a synthetic peptide, e.g., the B-chain is made by solid-phase synthesis. In a preferred embodiment, the B-chain is solubilized in urea.

40 In another preferred embodiment, the autoantigen is GAD65 or an immunologically active fragment or variant thereof.

45 In another preferred embodiment, the autoantigen is islet tyrosine phosphatase ICA512/IA-2 or an immunologically active fragment, or variant thereof.

50 In a preferred embodiment, the autoantigen is HSP60 or an immunologically active fragment or variant thereof.

In a preferred embodiment, the autoantigen is carboxypeptidase H or an immunologically active fragment or variant thereof.

In another preferred embodiment, the autoantigen is peripherin or an immunologically active fragment or variant thereof.

5 In yet another preferred embodiment, the autoantigen is a ganglioside, e.g., GM1-2, GT3, GD3, GM-1 or an immunologically active fragment or variant thereof.

10 In a preferred embodiment, the immunologically active fragment or variant of an autoantigen described herein lacks one or more biological activity of the native autoantigen, but retains the ability to react with an autoantigen antibody. E.g., an insulin fragment or variant lacks a hypoglycaemic effect.

In a preferred embodiment, the composition is a pharmaceutical composition.

In a preferred embodiment, the composition is a vaccine.

15 In a preferred embodiment, the oil-based adjuvant is IFA, Montanide ISA (e.g., Montanide ISA51) or an equivalent composition.

20 In a preferred embodiment, the autoantigen or fragment or variant is not recombinant. For example, the autoantigen is a synthetic peptide, e.g., made by solid-phase synthesis.

25 In a preferred embodiment, the subject is not immunocompromised, e.g., the subject is not HIV positive or does not have AIDS.

20 In another aspect, the invention features methods for the prevention or treatment of diabetes mellitus. The methods include, optionally, identifying a subject, e.g., a human, in need of prevention or treatment of the autoimmune disorder; and administering to the subject a composition, e.g., a vaccine, comprising a diabetes type 1 autoantigen and an oil-based carrier, e.g., an oil-based adjuvant, e.g., IFA or other oil based adjuvant, e.g., Montanide ISA51 or an equivalent composition. In preferred embodiments, the oil-based carrier or adjuvant, and preferably the composition, does not include a bacterial component, e.g., a mycobacterial component.

25 In a preferred embodiment, the autoantigen is an islet cell autoantigen.

30 In a preferred embodiment, the autoantigen is preproinsulin or an immunologically active fragment thereof, e.g., human insulin A-chain, B-chain, C peptide or an

immunologically active fragment or variant thereof, e.g., the autoantigen is a fragment of the sequence shown as SEQ ID NO:1.

In a preferred embodiment, the autoantigen is human insulin B-chain (amino acids 25-54 of SEQ ID NO:1) or an immunologically active fragment or variant thereof (e.g., residues 33-47 of SEQ ID NO:1). In a preferred embodiment, the B-chain or fragment thereof is not recombinant. For example, the B-chain or immunogenic fragment or variant thereof is a synthetic peptide, e.g., the B-chain is made by solid-phase synthesis. In a preferred embodiment, the B-chain is solubilized in urea.

In another preferred embodiment, the autoantigen is GAD65 or an immunologically active fragment or variant thereof.

In another preferred embodiment, the autoantigen is islet tyrosine phosphatase ICA512/IA-2 or an immunologically active fragment or variant thereof, e.g., amino acids 600-979 of SEQ ID NO:3 or an immunogenic fragment or variant thereof.

In a preferred embodiment, the autoantigen is HSP60 or an immunologically active fragment or variant thereof.

In a preferred embodiment, the autoantigen is carboxypeptidase H or an immunologically active fragment or variant thereof.

In another preferred embodiment, the autoantigen is peripherin or an immunologically active fragment or variant thereof.

In yet another preferred embodiment, the autoantigen is a ganglioside, e.g., GM1-2, GT3, GD3, GM-1 or an immunologically active fragment or variant thereof.

In a preferred embodiment, the autoimmune disorder is type 1 diabetes mellitus.

In a preferred embodiment, the autoantigen is human insulin B-chain.

In a preferred embodiment, the pharmaceutical composition is a vaccine.

In a preferred embodiment, the oil-based adjuvant is Montanide ISA (e.g., Montanide ISA51) or an equivalent composition.

In a preferred embodiment, the subject is not immunocompromised, e.g., the subject is not HIV positive or does not have AIDS.

In another aspect, the invention features a pharmaceutical composition, e.g., a vaccine, containing an autoantigen, e.g., a human autoantigen, e.g., a type 1 diabetes

autoantigen, and an oil-based carrier, e.g., IFA or other oil based adjuvant, e.g., Montanide ISA (e.g., Montanide ISA51) or an equivalent composition. In preferred embodiments, the oil-based carrier or adjuvant, and preferably the composition, does not include a bacterial component, e.g., a mycobacterial component.

5 In a preferred embodiment, the autoantigen is a diabetes type 1 autoantigen, e.g., an islet cell autoantigen.

In a preferred embodiment, the autoantigen is preproinsulin or an immunologically active fragment thereof, e.g., human insulin A-chain, B-chain, C peptide or an immunologically active fragment or variant thereof, e.g., the autoantigen is a fragment of the 10 sequence shown as SEQ ID NO:1.

In a preferred embodiment, the autoantigen is human insulin B-chain (amino acids 25-54 of SEQ ID NO:1) or an immunologically active fragment or variant thereof. In a preferred embodiment, the B-chain or fragment thereof is not recombinant. For example, the B-chain or immunogenic fragment or variant thereof is a synthetic peptide, e.g., made by solid-phase synthesis. In a preferred embodiment, the B-chain is solubilized in urea.

In another preferred embodiment, the autoantigen is GAD65 or an immunologically active fragment or variant thereof.

In another preferred embodiment, the autoantigen is islet tyrosine phosphatase ICA512/IA-2 or an immunologically active fragment or variant thereof, e.g., amino acids 600-979 of SEQ ID NO:3 or an immunogenic fragment or variant thereof.

In a preferred embodiment, the autoantigen is HSP60 or an immunologically active fragment or variant thereof.

In a preferred embodiment, the autoantigen is carboxypeptidase H or an immunologically active fragment or variant thereof.

25 In another preferred embodiment, the autoantigen is peripherin or an immunologically active fragment or variant thereof.

In yet another preferred embodiment, the autoantigen is a ganglioside, e.g., GM1-2, GT3, GD3, GM-1 or an immunologically active fragment or variant thereof.

In a preferred embodiment, the autoimmune disorder is type 1 diabetes mellitus.

30 In a preferred embodiment, the autoantigen is human insulin B-chain.

In a preferred embodiment, the pharmaceutical composition is a vaccine.

In a preferred embodiment, the adjuvant is Montanide ISA51 or an equivalent composition.

In a preferred embodiment, the immunologically active fragment or variant of an autoantigen described herein lacks one or more biological activity of the native autoantigen, 5 but retains the ability to react with an autoantigen antibody. E.g., an insulin fragment or variant lacks a hypoglycaemic effect.

The invention also features a pharmaceutical composition, e.g., a vaccine, comprising a diabetes type 1 autoantigen and an oil-based carrier, e.g., IFA or other oil based adjuvant, 10 e.g., Montanide ISA (e.g., Monatnide ISA51), where the diabetes type 1 autoantigen is in a dosage sufficient to provide a therapeutic effect in a human. In preferred embodiments, the oil-based carrier or adjuvant, and preferably the composition, does not include a bacterial component, e.g., a mycobacterial component.

In a preferred embodiment, the autoantigen is preproinsulin or an immunologically active fragment thereof, e.g., human insulin A-chain, B-chain, C peptide or an immunologically active fragment or variant thereof, e.g., the autoantigen is a fragment of the sequence shown as SEQ ID NO:1.

In a preferred embodiment, the autoantigen is human insulin B-chain or an immunologically active fragment or variant thereof.

In a preferred embodiment, the B-chain is solubilized in urea.

In a preferred embodiment, the B-chain or fragment thereof is not recombinant. For example, the B-chain or immunogenic fragment or variant thereof is a synthetic peptide, e.g., the B-chain is made by solid-phase synthesis.

In a preferred embodiment, the B-chain is solubilized in urea

25 In another preferred embodiment, the autoantigen is GAD65 or an immunologically active fragment or variant thereof.

In another preferred embodiment, the autoantigen is islet tyrosine phosphatase ICA512/IA-2 or an immunologically active fragment or variant thereof.

30 In a preferred embodiment, the autoantigen is HSP60 or an immunologically active fragment or variant thereof.

In a preferred embodiment, the autoantigen is carboxypeptidase H or an immunologically active fragment or variant thereof.

In another preferred embodiment, the autoantigen is peripherin or an immunologically active fragment or variant thereof.

5 In yet another preferred embodiment, the autoantigen is a ganglioside, e.g., GM1-2, GT3, GD3, GM-1 or an immunologically active fragment or variant thereof.

In a preferred embodiment, the autoimmune disorder is type 1 diabetes mellitus.

In a preferred embodiment, the human insulin B-chain is between 30-70%, preferably between 40-60%, more preferably between 45-55% proportion weight by weight (w/w).

10 In a preferred embodiment, the IFA or other oil based adjuvant is present between 30-70%, preferably between 40-60%, more preferably between 45-55% proportion weight by weight (w/w).

In a preferred embodiment, the human insulin B-chain and the IFA or other oil based adjuvant are present in about a 50/50 weight by weight ratio.

15 In a preferred embodiment, the human insulin B-chain is denatured, e.g., solubilized in urea and DTT.

In a preferred embodiment, the pharmaceutical composition is free of contaminants, e.g., pyrogens.

20 In a preferred embodiment, the immunologically active fragment or variant of an autoantigen described herein lacks one or more biological activity of the native autoantigen, but retains the ability to react with an autoantigen antibody. E.g., an insulin fragment or variant lacks a hypoglycaemic effect.

25 In another aspect, the invention features kits for preventing or treating an autoimmune disorder, e.g., diabetes mellitus, or other autoimmune disorder described herein. The kits contain a human autoantigen, e.g., a diabetes autoantigen described herein, an oil based carrier, e.g., an oil-based adjuvant, e.g., IFA, or other oil based adjuvant, e.g., Montanide ISA (e.g., Monatnide ISA51), and instructions indicating suitability for human use. In preferred embodiments, the oil-based carrier or adjuvant, and preferably the composition, does not 30 include a bacterial component, e.g., a mycobacterial component.

In a preferred embodiment, the kits contain a diabetes type 1 autoantigen, e.g., a diabetes type 1 autoantigen described herein; IFA or other oil based adjuvant, e.g., Montanide ISA (e.g., Monatnide ISA51); and instructions indicating suitability for human use, e.g., to prevent, delay the clinical onset of, or treat type 1 diabetes in a human.

5 In a preferred embodiment, the autoantigen is a synthetic peptide.

In a preferred embodiment, the autoantigen is lyophilized.

In a preferred embodiment, the autoantigen is preproinsulin or an immunologically active fragment thereof, e.g., human insulin A-chain, B-chain, C peptide or an immunologically active fragment or variant thereof, e.g., the autoantigen is a fragment of the sequence shown as SEQ ID NO:1.

In a preferred embodiment, the autoantigen is human insulin B-chain (amino acids 25-54 of SEQ ID NO:1) or an immunologically active fragment or variant thereof. In a preferred embodiment, the B-chain or fragment thereof is not recombinant. For example, the B-chain or immunogenic fragment or variant thereof is a synthetic peptide, e.g., made by solid-phase synthesis. In a preferred embodiment, the B-chain is solubilized in urea.

In another preferred embodiment, the autoantigen is GAD65 or an immunologically active fragment or variant thereof.

In another preferred embodiment, the autoantigen is islet tyrosine phosphatase ICA512/IA-2 or an immunologically active fragment or variant thereof.

In a preferred embodiment, the autoantigen is HSP60 or an immunologically active fragment or variant thereof.

In a preferred embodiment, the autoantigen is carboxypeptidase H or an immunologically active fragment or variant thereof.

In another preferred embodiment, the autoantigen is peripherin or an immunologically active fragment or variant thereof.

In yet another preferred embodiment, the autoantigen is a ganglioside, e.g., GM1-2, GT3, GD3, GM-1 or an immunologically active fragment or variant thereof.

In a preferred embodiment, the adjuvant is Montanide ISA51 or an equivalent composition.

In a preferred embodiment, the immunologically active fragment or variant of an autoantigen described herein lacks one or more biological activity of the native autoantigen,

but retains the ability to react with an autoantigen antibody. E.g., an insulin fragment or variant lacks a hypoglycaemic effect.

5 In another aspect, the invention features a delivery device, e.g., a syringe containing a composition described herein, e.g., an autoantigen, e.g., a diabetes type 1 autoantigen described herein; and an oil-based carrier, e.g., an adjuvant, e.g., IFA or other oil based adjuvant, e.g., Montanide ISA (e.g., Montanide ISA51). The delivery device, e.g., the syringe, can be configured for injection, e.g., intramuscular injection, in a human. In preferred embodiments, the oil-based carrier or adjuvant, and preferably the composition, 10 does not include a bacterial component, e.g., a mycobacterial component.

In a preferred embodiment, the adjuvant in the syringe is Montanide ISA51 or an equivalent composition.

15 In another aspect, the invention features a composition, e.g., a pharmaceutical composition, made by the method of: combining human insulin B-chain or an immunologically active fragment or variant thereof and an oil-based carrier, e.g., an oil-based adjuvant, e.g., IFA, or other oil based adjuvant, e.g., Montanide ISA (e.g., Montanide ISA51), and emulsifying the insulin B-chain and oil-based adjuvant. In a preferred embodiment, human insulin B-chain and oil-based adjuvant are combined in a weight by weight ratio (w/w) of between 30/70 to 70/30, preferably between 40/60 to 60/40, more preferably about a 50/50 w/w ratio. In a preferred embodiment, emulsification is performed with a high pressure syringe. In preferred embodiments, the oil-based carrier or adjuvant, and preferably the composition, does not include a bacterial component, e.g., a mycobacterial component.

20 25 In a preferred embodiment, the adjuvant is Montanide ISA51 or an equivalent composition.

In a preferred embodiment, the pharmaceutical composition is a vaccine.

30 In a preferred embodiment, the immunologically active fragment or variant of B-chain lacks one or more biological activity of the native autoantigen (e.g., lacks hypoglycaemic effect), but retains the ability to react with an autoantigen antibody.

In another aspect, the invention features a method of enabling a health care provider to treat an autoimmune disorder in a human subject. The method includes providing the health care provider with a human autoantigen or immunogenic fragment or variant thereof (e.g., a diabetes type 1 autoantigen described herein); optionally providing the health care provider with an oil-based carrier, e.g., an oil-based adjuvant, e.g., IFA or other oil based adjuvant, e.g., Montanide ISA (e.g., Montanide ISA51); and providing the health care provider with instructions for use of the autoantigen to treat the subject. In preferred embodiments, the oil-based carrier or adjuvant, and preferably the composition, does not include a bacterial component, e.g., a mycobacterial component.

10 In a preferred embodiment, the autoantigen is preproinsulin, GAD 65, ICA512/IA-2, HSP60, carboxypeptidase H, peripherin, and ganglioside.

15 In a preferred embodiment, the autoantigen is human insulin B-chain or immunogenic fragment or variant thereof.

20 In a preferred embodiment, the autoantigen is a synthetic peptide.

25 In a preferred embodiment, the human diabetes autoantigen is lyophilized.

30 In a preferred embodiment, the adjuvant is IFA.

In a preferred embodiment, the adjuvant is Montanide ISA51 or an equivalent composition.

In a preferred embodiment, the subject is not immunocompromised, e.g., the subject is not HIV positive or does not have AIDS.

In another aspect, the invention features a method of preparing a diabetes type 1 composition, e.g., a vaccine. The method includes (a) solubilizing a diabetes type 1 autoantigen, e.g., preproinsulin or an immunologically active fragment or variant thereof, e.g., insulin B-chain or an immunogenically active fragment or variant thereof, in urea and (b) emulsifying the solubilized autoantigen, e.g., B-chain, with an oil-based carrier, e.g., an oil-based adjuvant, e.g., IFA or other oil based adjuvant, e.g., Montanide ISA (e.g., Montanide ISA51),.

In a preferred embodiment, the B-chain or immunogenically active fragment, or variant thereof is a synthetic peptide.

In a preferred embodiment, the B-chain or immunogenically active fragment or variant thereof is solubilized in about 3 M to 5 M urea, preferably in about 3.5 M to 4.5 M urea, most preferably in about 4 M urea. Preferably, the B-chain is solubilized in the presence of a reducing agent, e.g., DTT or an equivalent reducing agent, e.g., 1 to 5 mg of DTT is added during the solubilization step.

In a preferred embodiment, the human insulin B-chain is present between 30-70%, preferably between 40-60%, more preferably between 45-55% proportion weight by weight (w/w) in the emulsification. In a preferred embodiment, the IFA is present between 30-70%, preferably between 40-60%, more preferably between 45-55% proportion w/w ratio. Most preferably, the human insulin B-chain and the IFA are present in a 50/50 w/w ratio.

In a preferred embodiment, emulsification is performed with high pressure sterile syringes.

In a preferred embodiment, the IFA is Montanide ISA51 or other IFA suitable for human use.

In another aspect, the invention features a method of evaluating the risk of onset of human type 1 diabetes in a subject. The method includes evaluating the levels of IFN- γ and/or IL-4 in a subject, e.g., serum IFN- γ and/or serum IL-4. The presence of serum IFN- γ and the absence of serum IL-4 correlates with a high incidence of developing diabetes. The presence of serum IL-4 (e.g., regardless of serum IFN- γ levels) correlates with a lower incidence of developing diabetes. IL-4 and IFN- γ serum levels can also be used, e.g., as markers to monitor the effect of a compound, e.g., an autoantigen vaccine described herein, on Th1/Th2 balance in the subject.

In a preferred embodiment, the levels of serum IFN- γ and/or serum IL-4 are evaluated by serum ELISA assay.

"Treatment" or "treating a subject" is defined as the application or administration of a therapeutic agent to a patient, or application or administration of a therapeutic agent to an isolated tissue or cell line from a patient, who has a disease, a symptom of disease or a predisposition toward a disease. Treatment can slow, cure, heal, alleviate, relieve, alter, remedy, ameliorate, improve or affect the disease, the symptoms of disease or the

predisposition toward disease. For example, treatment of a subject, e.g., a human subject, with a composition described herein, can slow, improve, or stop the ongoing autoimmunity, e.g., a reaction against pancreatic β -cells, in a subject before, during, or after the clinical onset of type 1 diabetes.

5 An "oil-based carrier" as used herein is a composition that includes at least 10% by weight of a natural or synthetic oil suitable for administration to a human in conjunction with a therapeutic agent. In preferred embodiments, the carrier includes at least 20, 30, 50, 70, 80, 90, 95, 98, or 99% oil by weight. In some embodiments, the oil-based carrier can include less than 70, 60, 50, 40, 30 or 20% oil by weight. In preferred embodiments, the oil will be 10 in the range of 10 to 95%, preferably 20 to 90% or 30 to 70% oil by weight. The oil should be chosen such that it provides for sustained release of a substance dispersed within it when administered to a subject. Suitable oils include mineral oil (e.g., Drakeol 6 VR light mineral oil), vegetable oil, squalene, or liquid paraffin. In some embodiments, the oil-based carrier can contain more than one type of oil. In some embodiments, the oil-based carrier can 15 include an immune stimulator, e.g., an immunostimulating glucan, but it is much preferred that the oil-based carrier does not include an immune stimulator, e.g., an immunostimulating glucan, a bacterial component, e.g., a mycobacterial component. In a preferred embodiment, the oil-based carrier does not include an alum component.

20 While not wanting to be bound by theory, an oil based carrier is believed to work by triggering the immunocompetent cells, which are related to the inflammatory ability. An oil-based carrier can also act as an antigen vehicle and a slow release or long-term antigen presentation device. When injected into a subject, an oil-based carrier and antigen 25 composition can form a depot of antigen at the injection site, thereby protecting the antigen from degradation. From this depot the antigen can be released slowly into the system and provides a prolonged antigen presentation as well as expanded total contact surface area and the attraction of inflammatory cells. Macrophages can digest most of the incorporated materials and present the processed antigens on their surface

Oil based carriers should include an emulsifier or surfactant component. The 30 emulsifier or surfactant (and the amount of emulsifier or surfactant) is chosen such that it facilitates the mixture or dispersion of a substance, e.g., an antigen preparation, with the oil. An oil-based carrier can include 0.1 to 50%, preferably 1 to 30%, more preferably 5 to 20%

by weight of a surfactant or emulsifier. Examples of emulsifiers or surfactants include Arlacel A, mannide oleate (e.g., Montanide 80-mannide monooleate), anhydrous mannitol/oleic acid ester, polyoxyethylene or polyoxypolyethylene.

An "autoantigen" as used herein, is a protein that despite being a normal cell or tissue constituent, can be the target of a humoral or cell-mediated immune response in a subject. For example, diabetes type 1 autoantigens are typically normal protein constituents of pancreatic cells. An "immunologically active fragment" of an autoantigen described herein is an autoantigen in which one or more amino acid residues have been deleted and the fragment retains the ability to react with an autoantigen antibody or to stimulate the production of antibodies against the autoantigen. For example, an immunologically active fragment can be an autoantigen polypeptide in which residues have been successively deleted from the amino- and/or carboxyl-termini, while substantially retaining immunogenic activity. For example, insulin B-chain (amino acids 25-54 of SEQ ID NO:1) is an immunologically active fragment of preproinsulin; a polypeptide that includes amino acids 33-37 of SEQ ID NO:1 is an immunologically active fragment of B-chain; a polypeptide that includes amino acids from about 600 to 979 of SEQ ID NO:3 includes an immunologically active fragment of IA-2. In a preferred embodiment, the immunologically active fragment is a fragment of any of SEQ ID Nos:1-6. Preferred fragments lacks one or more biological activities of the native autoantigen, but retain the ability to react with an autoantigen antibody. E.g., a preferred insulin fragment or variant lacks a hypoglycaemic effect. Preferably, an immunologically active fragment of an autoantigen described herein is between 4 and 400 amino acid residues in length, more preferably between 10 and 300 amino acid residues in length, more preferably between 30 and 200 amino acid residues in length.

An "immunologically active variant" of an autoantigen described herein is an autoantigen that has been modified by addition, modification or substitution of one or more amino acid residues in the naturally occurring autoantigen and retains the ability to react with an autoantigen antibody or to stimulate the production of antibodies against the autoantigen. The variants described herein encompass allelic and polymorphic variants, and also muteins and fusion proteins that retain the ability to bind an autoantigen antibody or to produce an immune response against the autoantigen in a human. For example, up to 20%, preferably up to 10%, of the amino acid residues of an autoantigen can be replaced with substitute amino

acids, so long as the variant retains the ability to bind autoantigen or produce an immune response against the autoantigen, e.g., in a human. A variant can also include an autoantigen or fragment thereof described herein in which one or more amino acids have been inserted or added, e.g., an autoantigen that has been coupled or fused to a carrier peptide. Also included are variants containing modifications, such as incorporation of unnatural amino acid residues, or phosphorylated, sulfonated, or biotinylated amino acid residues. Modifications of amino acid residues may also include aliphatic esters or amides of the carboxyl terminus or of residues containing carboxyl side chains, O-acyl derivatives of hydroxyl group-containing residues, and N-acyl derivatives of the amino-terminal amino acid or amino-group containing residues, e.g., lysine or arginine. Other modifications include the addition of other moieties, particularly those that may increase the immunogenicity of the autoantigen. Preferred variants lack one or more biological activities of the native autoantigen, but retain the ability to react with an autoantigen antibody. E.g., a preferred insulin variant lacks a hypoglycaemic effect.

All publications cited herein are hereby incorporated by reference in their entirety. The details of one or more embodiments of the invention are set forth in the accompanying drawings and the description below. Other features, objects, and advantages of the invention will be apparent from the description and drawings, and from the claims.

DETAILED DESCRIPTION

The present invention relates to methods of preventing or treating an autoimmune disorder, e.g., Type 1 diabetes mellitus, in humans. The method includes administering to a patient a pharmaceutical composition, e.g., a vaccine, containing an autoantigen, e.g., a type 1 diabetes autoantigen described herein, in IFA, or other oil-based adjuvant, e.g., Montanide ISA51 or an equivalent composition. A preferred adjuvant includes an oil and an emulsifier. In a preferred embodiment, the vaccine can stop or slow down the ongoing autoimmunity, e.g., a reaction against pancreatic β -cells at the clinical onset of the disease. Although not wishing to be bound by theory, it is believed that the vaccine can alter the autoimmune process and stop autodestruction of the pancreatic β -cells.

Incomplete Freund's adjuvant (IFA) is a preferred delivery vehicle for the autoantigen in humans. The methods of the invention can prevent diabetes mellitus, or prevent or delay

loss of residual β -cell mass, providing a longer remission period and delaying the onset of diabetes related, usually progressive, complications at a later stage of the life.

Autoantigens

5 Autoantibodies against insulin, glutamic acid decarboxylase (GAD) and other islet cell autoantigens, e.g., ICA 512/IA-2 protein tyrosine phosphatase, ICA12, ICA69, are frequently found in newly diagnosed diabetic patients. Thus, type 1 diabetes autoantigens useful in the methods of the invention include, e.g., preproinsulin or an immunologically active fragment thereof (e.g., insulin B-chain, A chain, C peptide or an immunologically active fragment thereof), and other islet cell autoantigens (ICA), e.g., GAD65, islet tyrosine phosphatase ICA512/IA-2, ICA12, ICA69 or immunologically active fragments thereof. Other type 1 diabetes autoantigens include HSP60, carboxypeptidase H, peripherin, gangliosides (e.g., GM1-2, GM3) or immunologically active fragments thereof. Any of the type 1 diabetes autoantigens described herein, or immunologically active fragments, analogs or derivatives thereof, are useful in the methods and compositions of the invention.

10 15 Autoantigens useful in treatment of other diseases include myelin proteins for prevention or treatment of multiple sclerosis or Type II collagen for prevention or treatment of rheumatoid arthritis.

20 Diabetes autoantigens

Preproinsulin

25 The insulin mRNA is translated as a 110 amino acid single chain precursor called preproinsulin, and removal of its signal peptide during insertion into the endoplasmic reticulum generates proinsulin. Proinsulin consists of three domains: an amino-terminal B chain, a carboxy-terminal A chain and a connecting peptide in the middle known as the C peptide. Within the endoplasmic reticulum, proinsulin is exposed to several specific endopeptidases which excise the C peptide, thereby generating the mature form of insulin which consists of the A and B chain. Insulin and free C peptide are packaged in the Golgi into secretory granules which accumulate in the cytoplasm. The preproinsulin peptide 30 sequence is as follows.

MALWMRLLPL LALLALWGPD PAAAFVNQHL CGSHLVEALY LVCGERGFFY TPKTRREAED
LQVGQVELGG GPGAGSLQPL ALEGSLQKRG IVEQCCTSIC SLYQLENYCN (SEQ ID NO:1)

5 Insulin A chain includes amino acids 90-110 of SEQ ID NO:1. B chain includes
amino acids 25-54 of SEQ ID NO:1. The connecting sequence (amino acids 55-89 of SEQ
ID NO:1) includes a pair of basic amino acids at either end. Proteolytic cleavage of
proinsulin at these dibasic sequences liberates the insulin molecule and free C peptide, which
includes amino acids 57-87 of SEQ ID NO:1. The human preproinsulin or an
10 immunologically active fragment thereof, e.g., B chain or an immunogenic fragment thereof,
e.g., amino acids 33-47 of SEQ ID NO:1 (corresponding to residues 9-23 of the B-chain), are
useful as autoantigens in the methods and compositions described herein.

GAD65

15 Gad65 is a primary β -cell antigen involved in the autoimmune response leading to
insulin dependent diabetes mellitus (Christgau et al. (1991) *J Biol Chem.* 266(31):21257-
64). The presence of autoantibodies to GAD65 is used as a method of diagnosis of type 1
diabetes. Gad65 is a 585 amino acid protein as follows (SEQ ID NO:2).

20 MASPGSGFW S FGSEDGSGDS ENPGTARAWC QVAQKFTGGI GNKLCALLYG DAEKPAESGG
SQPPRAAARK AACACDQKPC SCSKVDVNYA FLHATDLLPA CDGERPTLAF LQDVMNILLQ
YVVKSFDRST KVIDFHYPNE LLQEYNWELA DQPQNLEEIL MHCQTTLKYA IKTGHPRYFN
QLSTGLDMVG LAADWLTSTA NTNMFTEIA PVFVLLEYVT LKKMREIIGW PGGSGDGIFS
PGGAISNMYA MMIARFKMFP EVKEKGMAAL PRLIAFTSEH SHFSLKKGAA ALGIGTDSVI
25 LIKCDERGKM IPSDLERRIL EAKQKGFVPF LVSATAGTTV YGAFDPLLAV ADICKKYKIW
MHVDAAWGGG LLMSRKHKWK LSGVERANSV TWNPBKMMGV PLQCSALLVR EEGLMQNCNQ
MHASYLFQQD KHYDLSYDTG DKALQCGRHV DVFKLWLMWR AKGTTGFEAH VDKCLELAEY
LYNIIKNREG YEMVFDGKPQ HTNVCFWYIP PSLRTLEDNE ERMSRLSKVA PVIKARMMEY
GTTMVSQPL GDKVNFFRMV ISNPAATHQD IDFLIEEIER LGQDL (SEQ ID NO:2)

30 *Islet tyrosine phosphatase IA-2*

IA-2/ICA512), a member of the protein tyrosine phosphatase family, is another major
autoantigen in type 1 diabetes (Lan et al. *DNA Cell Biol* 13:505-514, 1994). 70% of diabetic
patients have autoantibodies to IA-2, which appear years before the development of clinical

disease. The IA-2 molecule (SEQ ID NO:3, below) is 979 amino acids in length and consists of an intracellular, transmembrane, and extracellular domain (Rabin et al. (1994) *J. Immunol.* 152 (6), 3183-3188). Autoantibodies are typically directed to the intracellular domain, e.g., amino acids 600-979 of SEQ ID NO:3 and fragments thereof (Zhang et al. (1997) *Diabetes* 46:40-43; Xie et al. (1997) *J Immunol* 159:3662-3667). The amino acid sequence of IA-2 is as follows.

5 MRRPRRPGGLGGSGGLRLLLCLLLSSRPCCSAVSAHGCLFDRLCSHLEVCIQDGLFG
 QCQVGVGQARPLLQVTSPVLQRLQGVLRQLMSQGLSWHDDLTQYVISQEMERIPRLRPPE
 10 10 PRPRDRSGLAPKRPGPAGELLQDIPGTGAPAAQHRLPQPPVGKGGAGASSSLPLQAEI
 LPPLLEHLLLPPQPPHPSLSYEPALLQPYLFHQFGSRDGSRVSEGSPGMVSGPLPKAEA
 PALFSRTASKGIFGDPGHSYGDLPGPSPAQLFQDSGLLYLAQELPAPSRARVPRLPEQG
 SSSRAEDSPEGYEKEGLGDRGEKPASPAVQPDAALQRLAAVLAGYVELRQLTPEQLSTL
 LTLLQLLPKGAGRNPAGVVNVGADIKKTMEGPVEGRDTAELPARTSPMPGHPTASPTSSE
 15 15 VQQVPSPVSEPPKAARPVTPVLLKKSPLGQSQPTVAGQPSARPAEEYGYIVTDQKP
 LSLAAGVKLLEILAEHVHMSSGSFINISVVGPAITFRIRHNEQNLSLADVTQQAGLVKSE
 LEAQTGLQILQTGVGQREAAAVLPQTAHSTSPMRSVLLTLVALAGVAGLLVALAVALCV
 RQHARQQDKERLAALGPEGAHGDFTFEYQDLCRQHMATKSLFNRAEGPPEPSRVSSVSSQ
 FSDAAQASPSSHSSTPSWCEEPQAQANMDISTGHMILAYMEDHLRNDRDLAKEWQALCAYQ
 20 20 AEPNTCATAQGEGNIKKNRHPDFLPYDHARIKLVESSPSRSDYINASPIIEHDPRMPAY
 IATQGPLSHTIADFWQMVWESGCTVIVMLTPLVEDGVKQCDRYWPDEGASLYHVYEVNLV
 SEHIWCEDFLVRSFYLKNVQTQETRTLTFQHFSLWPAEGTPASTRPLLDFRRKVNKCYRG
 RSCPIIVHCSDGAGRTGYILIDMVLRMAKGVKEIDIAATLEHVRDQRPGVRSKDQFEF
 ALTAVAEEVNAILKALPQ (SEQ ID NO:3)

25

ICA12

ICA 12 (Kasimiotis et al. (2000) *Diabetes* 49(4):555-61; Gen bank Accession No. AAD16237; SEQ ID NO:4) is one of a number of islet cell autoantigens associated with diabetes. The sequence of ICA12 is as follows.

30 MSMRSPISAQ LALDGVTMV NCTIKSEEKK EPCHEAPQGS ATAAEPQPGD PARASQDSAD
 PQAPAQGNFR GSWDCSSPEG NGSPEPKRPG ASEASGSQE KLDFNRLKE VVPAIEKLLS
 SDWKERFLGR NSMEAKDVKG TQESLAEKEL QLLVMIHQLS TLRDQLLTQH SEQKNMAAML

FEKQQQQMEL ARQQQEQUIAK QQQLIQQQH KINLLQQQIQ QVNMPYVMIP AFPPSHQPLP
 VTPDSQLALP IQPIPCKPVE YPLQLLHSPP APVVKRPGAM ATHHPLQEPS QPLNLTAKPK
 APELPNTSSS PSLKMSSCVP RPPSHGGPTR DLQSSPPSLP LGFLGEGDAV TKAIQDARQL
 5 LSHSGALDG SPNTPFRKDL ISLDSSPAKE RLEDGCVHPL EEAMLSCDMG GSRHFPESRN
 SSHIKRPMNA FMWAKDERR KILQAFPDMH NSSISKILGS RWKSMTNQEK QPYYEEQARL
 SRQHLEKYPD YKYKPRPKRT CIVEGKRLRV GEYKALMRTR RQDARQSYVI PPQAGQVQMS
 SSDVLYPRAA GMPLAQPLVE HYVPRSLDPN MPVIVNTCSL REEGEGTDDR HSVADGEMYR
 YSEDEDSEGE EKSDGELVVL TD (SEQ ID NO:4)

10 *ICA69*

ICA69 is another autoantigen associated with type 1 diabetes (Pietropaolo et al. J Clin Invest 1993; 92:359-371). The amino acid sequence of ICA69 is as follows.

MSGHKCSYPW DLQDRYAQDK SVVNKMQQRY WETKQAFIKA TGKKEDEHVV ASDADLDAKL
 15 ELFHSIQRTC LDLSKAIVLY QKRICFLSQE ENELGKFLRS QGFQDKTRAG KMMQATGKAL
 CFSSQQRLAL RNPLCRFHQE VETFRHRAIS DTWLTVNRME QCRTEYRGAL LWMKDVSQEL
 DPDLYKQMEK FRKVQTQVRL AKKNFDKLKM DVCQKV DLLG ASRCNLLSHM LATYQTTLLH
 FWEKTSHTMA AIHESFKGYQ PYEFTTLKSL QDPMKKLVEK EKKKINQQE STDAAVQEPS
 20 QLISLEENQ RKESSSFKTE DGKSILSALD KGSTHTACSG PIDELLDMKS EEGACLGPVA
 GTPEPEGADK DDLLLSEIF NASSLEEGERF SKEWAAVFGD GQVKEPVPTM ALGEPDPKAQ
 TGSGFLPSQL LDQNMKDLQA SLQEPAKAAS DLTAWFSLFA DLDPLSNPDA VGKTDKEHEL
 LNA (SEQ ID NO:5)

glima38

Glima 38 is a 38 kDa islet cell membrane autoantigen which is specifically immunoprecipitated with sera from a subset of prediabetic individuals and newly diagnosed type 1 diabetic patients. Glima 38 is an amphiphilic membrane glycoprotein, specifically expressed in islet and neuronal cell lines, and thus shares the neuroendocrine expression patterns of GAD65 and IA2 (Aanstoot et al. J Clin Invest. 1996 Jun 15;97(12):2772-2783).

30 *Heat shock protein 60 (HSP60)*

HSP60, e.g., an immunologically active fragment of HSP60, e.g., p277 (see Elias et al., Eur J Immunol 1995 25(10):2851-7), can also be used as an autoantigen in the methods

and compositions described herein. Other useful epitopes of HSP 60 are described, e.g., in U.S. Patent No. 6,110,746.

5 *Carboxypeptidase H*

Carboxypeptidase H has been identified as an autoantigen, e.g., in pre-type 1 diabetes patients (Castano et al. (1991) *J Clin Endocrinol Metab* 73(6):1197-201; Alcalde et al. *J Autoimmun.* 1996 Aug;9(4):525-8.). Therefore, carboxypeptidase H or immunologically reactive fragments thereof (e.g., the 136-amino acid fragment of carboxypeptidase-H described in Castano, *supra*) can be used in the methods and compositions described herein.

10

15 *Peripherin*

Peripherin is a 58 KDa diabetes autoantigen identified in nod mice (Boitard et al. (1992) *Proc Natl Acad Sci U S A* 89(1):172-6. The human peripherin sequence is shown as SEQ ID NO:6, below.

15 MSHHPGLRA GFSSTSRYRT FGPPPSLSPG AFSYSSSSRF SSSRLLGSAS PSSSVRLGSF
 RSPRAGAGAL LRLPSERLDF SMAEALNQEF LATRSNEKQE LQELNDRFAN FIEKVRFLEQ
 QNAALRGELS QARGQEPEARA DQLCQQLRE LRRELELLGR ERDRVQVERD GLAEDLAALK
 QRLEEETRKR EDAEHNVLFL RKDVVDDATLS RLELERKIES LMDEIEFLKK LHEEELRDLQ
 VSVESQQVQQ VEVEATVKPE LTAALRDIRA QYESIAAKNL QEAEWYKSK YADLSDAANR
 NHEALRQAKQ EMNESRRQIQ SLTCEVDGLR GTNEALLRQL RELEEQFALE AGGYQAGAAR
 LEEELRQLKE EMARHLREYQ ELLNVKMAKD IEIATYRKLL EGEESRISVP VHSFASLNIK
 TTVPEVEPPQ DSHSRKTVLI KTIETRNGEQ VVTESQKEQR SELDKSSAHS Y (SEQ ID
 NO:6)

20 25 *Gangliosides*

Gangliosides can also be useful autoantigens in the methods and compositions described herein. Gangliosides are sialic acid-containing glycolipids which are formed by a hydrophobic portion, the ceramide, and a hydrophilic part, i.e. the oligosaccharide chain.

30 Gangliosides are expressed, inter alia, in cytosol membranes of secretory granules of pancreatic islets. Auto-antibodies to gangliosides have been described in type 1 diabetes, e.g., GM1-2 ganglioside is an islet autoantigen in diabetes autoimmunity and is expressed by human native β cells (Dotta et al. *Diabetes*. 1996 Sep;45(9):1193-6). Gangliosides GT3, GD3 and GM-1 are also the target of autoantibodies associated with autoimmune diabetes (reviewed in Dionisi et al. *Ann Ist Super Sanita* 1997;33(3):433-5). Ganglioside GM3

participates in the pathological conditions of insulin resistance (Tagami et al. *J Biol Chem* 2001 Nov 13; online publication ahead of print).

Multiple Sclerosis Autoantigens

5 Autoantigens thought to be involved in multiple sclerosis (MS) include myelin basic protein, myelin oligodendrocyte glycoprotein, and human transaldolase.

Rheumatoid Arthritis Autoantigens

10 Collagen type II and cartilage proteins YKL-39 (Sekine et al. (2001) *Annals of the Reumatic Diseases* 60:49-54) and YKL-40 (Kawasaki et al. (2001) *J Rheumatol* 28:341-345) have been reported to be autoantigens in rheumatoid arthritis.

15 Reintroduction of autoantigen in autoimmune disease can generate protective antigen-specific cell mediated immunity. A number of regulatory mechanisms differentially regulate the autoaggressive Th1 and protective Th2 cell response (the two subsets of T helper cells) in mice and human alike. Th1 and Th2 have distinct and defined cytokine secretion profiles. The antigen activated Th1 (autoaggressive) cells release IL-2 and IFN- γ , which inhibit the Th2 (protective) cell production of IL-4 and IL-10. Insulin B-chain in IFA has been shown to reduce IFN- γ (Th1) expression and reduce insulitis (Muir et al. (1995) *J Clin Invest* 95:628-634; Orban et al. (1999) *Diabetes* 48 Supp.1:A216-A217). Nasal administration of GAD65 also leads to change in Th1/Th2 balance in favor of Th2 'protective' cells response (Tian et al. (1996) *J Exp Med* 183:1561-1567). GAD in incomplete Freund's adjuvant in NOD mice leads to shift of Th1/Th2 balance toward the production of Th2 related cytokines like IL-4 and IL-10 (Sai et al. (1996) *Clin Exp Immunol* 105:330-337). Secretion of IgG1, IgG4 and IgE antibodies are preferentially stimulated by conditions leading to selective activation of 20 Th2 lymphocytes (IL-4, IL-10 and the absence of IFN-gamma).

25 There are many autoantigens considered to be important in human Type 1 diabetes mellitus. Several data suggest that insulin is a major antigen playing roles in the pathogenesis of the disease (Muir et al. (1993) *Diabetes Metab Rev* 9:279-287). Insulin, a β -cell specific major protein is moderately immunogenic when used alone, and has been shown 30 in a pilot human trial to have the effect of delaying the development of diabetes mellitus (Keller et al. (1993) *Lancet* 341:927-928). However, it must be injected daily over long

periods of time to induce the desired effect. When insulin is used in humans, there is always a major concern about hypoglycemia and its sequels.

5 Immunogenic fragments or variants of insulin or preproinsulin lacking hypoglycaemic effect are a safe choice for human use. For example, insulin B-chain (or immunogenic fragments and variants thereof) can be used as an immune modulator to prevent or delay further loss of functional, residual β -cell mass, after the clinical onset of Type 1 diabetes in humans, without hypoglycaemic effect. The reintroduction of autoantigen, e.g., insulin B-chain, in human subjects can act to change to autoimmune process triggering a protective immune response. The Th1/Th2 balance can change in favor 10 of a protective Th2 type cell response.

Human insulin B-chain for human vaccine use can be made by a standard solid-phase peptide synthesis (Example 1). A procedure for effective solubilization of the insulin B-chain is described herein (Example 2).

15 Changes in autoantibody titers and in GAD65AB isotypes reflecting the effect of autoantigen vaccination can be used to characterize the regression in autoimmune process in diabetic or prediabetic patients. In addition, there will be changes in stimulated cytokine profile (in favor of Th2-cells) correlating with the effect of the autoantigen vaccination, which later may be used as cellular marker for regression of autoimmunity in Type 1 diabetes mellitus.

Animal Studies

20 To test the efficacy of autoantigens in IFA, NOD mice were vaccinated with human recombinant GAD65 (300 μ g/mice) in IFA (total volume 0.1 ml), human insulin B-chain (20.1 μ g/mice) in IFA (total volume 0.1 ml) and sham protein (Lysozyme, 200 μ g/mice) in IFA (total volume 0.1 ml) sc. in the inguinal areas at 3, 5 and 7 weeks of age. At 9 weeks of age the treated mice were sacrificed and their splenocytes (1.0×10^7 cells) were cotransferred with splenocytes from 9 weeks old untreated NOD mice (1.0×10^7 cells) into 9-11 weeks old NOD scid/scid mice (16 mice/group; total 2.0×10^7 cells/mice). Control group received splenocytes (2.0×10^7 cells/mice) from untreated NOD mice. By the 75th day after adoptive 25 transfer all the scid/scid mice, recipients of splenocytes from the control and the sham protein vaccinated donor mice developed diabetes. 4/16 scid/scid mice, recipient of splenocytes

10276-067001 (JDP-067)

from the GAD and 9/16 from the B-chain treated donor mice cotransferred with untreated mice splenocytes became diabetic ($p=0.0001$ and $p=0.0005$ respectively compared to the sham protein vaccinated group).

Histological studies were performed on the pancreases of the vaccinated mice at the time of adoptive transfer. The insulitis were graded on a 0-4 scale as previously described (Charlton et al. (1989) *Diabetes* 38:441-447). The mean insulitis scores were 1.31 in the GAD65 and 1.20 in B-chain treated groups meanwhile 2.64 in the sham protein treated and 2.71 in the control group ($p=0.0001$ for both GAD65 and B-chain vaccinated groups compare to sham protein treated mice).

These data show that immunization with a diabetes type 1 autoantigen, e.g., insulin B-chain or GAD65 protein, in IFA significantly reduces the incidence of diabetes in NOD scid/scid adoptive transfer model. The vaccines generated changes in the immune competent cells, which actively suppress the capacity of T-cells from prediabetic untreated donors to transfer the disease adoptively. The vaccination with these autoantigens also reduced insulitis in NOD mice. Sham protein in IFA was not effective, thus the intervention is antigen specific. Adjuvant enhanced autoantigen vaccination, e.g., with insulin B-chain or GAD65, actively suppressed the ongoing autoimmunity in mice thus provides diabetes-specific therapeutic intervention. Similar data on a different preparation of insulin B-chain have been reported by Muir et al. (1995, *J. Clin. Invest.* 95:628-634). Data on safety for human are summarized herein below.

Human studies

Assays were developed to measure human GAD65 autoantibody isotypes. The data indicate that individuals with diabetes autoimmunity, but long diabetes-free follow up have a higher Th2 related GAD65 specific IgG isotypes measured by IgG1/IgG2 or by IgG1+IgG4/IgG2+IgG3 ratios than the aged matched newly diagnosed patients with Type 1 diabetes mellitus (1.15 ± 0.08 SE vs. 0.76 ± 0.09 SE $p=0.006$ and $1.11 \pm$ SE vs. 0.78 ± 0.07 SE $p=0.002$ respectively). This is indirect evidence that Th1/Th2 balance is at play in the pathogenesis of human Type 1 diabetes and consistent with Th1 dominance driving towards diabetes meanwhile Th2 activity contra-acts this process. This marker can be used to monitor the effect of the IFA enhanced autoantigens on the Th1/Th2 balance.

Many years before clinical onset of Type 1 diabetes, islet cell antibodies (ICA) can be detected. A subgroup of these ICA-positive individuals does not develop diabetes after long-term follow-up. We have studied 17 such ICA positive individuals with an average of 8.8 (3.7 to 29.2) years diabetes-free observation period. Fifteen have two or more samples

5 collected at different time, from 1.6 to 8.2 years. All patients have autoantibodies against GAD65 human recombinant protein (positive index >0.1), 15/17 in high titer (index>0.5). This is significant increase compared to 19 ICA positive patients with diabetes (10/19 GAD65 positive index>0.5 -high titer, p=0.01). They were also positive for IA2 or insulin autoantibodies. We have further studied this group of high-risk for Type 1 diabetes, non-
10 progressors. CD4- CD8- double negative V α 24J α Q $^+$ T cells were isolated from these individuals and 33 clones raised secreted IL-4 and IFN-gamma on stimulation with anti-CD3. This secretory pattern, which was similar to healthy controls, was in sharp contrast to patients with new onset Type 1 diabetes. None of the 56 clone tested from newly diagnosed patients with Type 1 diabetes secreted IL-4, thus showing an extreme Th1 bias. Seven of the 14 high-risk for Type 1 diabetes, non-progressor individuals had IL-4 level >1ng/ml detectable by
15 ELISA in their serum. When compared to either healthy or new onset Type 1 diabetes mellitus patients the frequency of serum IL-4 positive individuals was significantly elevated in the non-progressor group. Consequently, we examined serum levels of IL-4 and IFN- γ activity using ELISA in subjects at high risk for developing Type 1 diabetes mellitus and correlated the results with disease outcome. A total of 1100 archived serum samples from
20 443 diabetes-free first degree relatives of patients with Type 1 diabetes mellitus were studied; 95 developed Type 1 diabetes during follow-up. Serum IL-4 and IFN- γ by ELISA and additional autoantibodies for GAD65 and for IA2 were measured. Subjects with serum IFN- γ ELISA activity (without IL-4 ELISA activity) levels had a significantly higher incidence of
25 developing diabetes after five years (74.0% vs. cytokine negatives p=0.03)), while subjects with serum IL-4 ELISA pattern, regardless of IFN- γ ELISA status, had a significantly lower incidence of developing diabetes (17.3% vs. cytokine negatives p=0.003).

Thus, levels of serum ELISA IL-4 and IFN- γ activities are predictors of the onset of human Type 1 diabetes. We found that serum autoantibodies are independent markers for
30 risk assessment in Type 1 diabetes.

These cellular (CD4- CD8- double negative V α 24J α Q $^+$ T cells) and humoral (IL-4

and IFN-gamma ELISA serum levels) markers described herein can be used to monitor the effect of the IFA enhanced autoantigens on the Th1/Th2 balance.

Oil-based Adjuvants

5 An oil-based adjuvant typically consists of two components: (1) an oil, and (2) an emulsifier or surfactant, mixed with water. Suitable oils and emulsifiers are known in the art. For example, the oil can be mineral oil, vegetable oil, squalene or liquid paraffin. The emulsifier or surfactant can be, e.g., Arlacel A, mannide oleate, anhydrous mannitol/oleic acid ester, polyoxyethylene or polyoxypropylene. Exemplary oil-based adjuvants include 10 conventional IFA, Montanide ISA adjuvants, or Hunter's TiterMax adjuvant. In preferred embodiments, the adjuvant includes 20 to 95%, preferably 30 to 90%, more preferably 40 to 70% by weight of an oil phase, and 0.1 to 50%, preferably 1 to 30%, more preferably 5 to 20% by weight of a surfactant or emulsifier. Various types of oil-based adjuvants are 15 described, e.g., in U.S. Patent No. 5,814,321, U.S. Patent No. 6,299,884, U.S. Patent No. 6,235,282, U.S. Patent No. 5,976,538, U.S. Patent No. 5,904.

IFA is typically a mixture of a non-metabolizable oil (e.g., mineral oil), a surfactant (e.g., Arlacel A). Unlike Complete Freund's Adjuvant (CFA), IFA does not contain a bacterial component, e.g., mycobacteria. The first large-scale vaccinations using IFA in humans took place on US military personnel (Davenport (1968) *Ann Allergy* 26:288-292; Beebe et al. (1972) *Am J Epidemiol* 95:337-346; Salk & Salk (1977) *Science* 195:834-847). The findings were essentially negative with respect to malignancy, allergic diseases and collagenosis, but there was evidence that some men had a cyst like reaction at the site of inoculation. From these experiments, IFA was regarded by some as unsuitable for human purposes, although it has remained widely used in animal research. In recent years, newer 20 forms of IFA have been shown safe for human use in HIV immunotherapy or therapeutic vaccinations (Turner et al. (1994) *AIDS* 8:1429-1435; Trauger et al. (1995) *J Acquir Immune Defic Syndr Hum Retrovirol* 10 Supp2:S74-82; Trauger et al. (1994) *J Infect Dis* 169:1256-1264).

Montanide ISA Adjuvants (Seppic, Paris, France) are a group of oil/surfactant based 30 adjuvants in which different surfactants are combined with either a non-metabolizable mineral oil, a metabolizable oil, or a mixture of the two. They are prepared for use as an

emulsion with aqueous Ag solution. The surfactant for Montanide ISA 50 (ISA = Incomplete Seppic Adjuvant) is mannide oleate, a major component of the surfactant in Freund's adjuvants. The surfactants of the Montanide group undergo strict quality control to guard against contamination by any substances that could cause excessive inflammation, as has 5 been found for some lots of Arlacel A used in Freund's adjuvant. The various Montanide ISA group of adjuvants are used as water-in-oil emulsions, oil-in-water emulsions, or water-in-oil-in-water emulsions. The different adjuvants accommodate different aqueous phase/oil phase ratios, because of the variety of surfactant and oil combinations.

10 Hunter's TiterMax (CytRx Corp., Norcross, GA) is an oil/surfactant-based adjuvant prepared as a water-in-oil emulsion in a manner similar to that used for conventional Freund's adjuvants. However, it uses a metabolizable oil (squalene) and a nonionic surfactant that has good protein antigen-binding capacity as well as adjuvant activity. The adjuvant activity may relate, in part, to the surfactant's ability to activate complement and bind complement components, as this helps target the Ag to follicular dendritic cells in the spleen 15 and lymph nodes. The surfactant used in the commercially available adjuvant is one of a number of synthetic nonionic block copolymers of polyoxyethylene and polyoxypropylene developed by Hunter (Hunter et al., 1991 *Vaccine* 9:250-256). The utilization of copolymer-coated microparticles to stabilize the emulsion permits formation of stable emulsions with less than 20% oil, an important factor in minimizing total adjuvant injected.

20 An adjuvant can be used with antigens to elicit cell-mediated immunity and the production of antibodies of protective isotypes (IgG2a in mice and IgG1 in primates). Different types of adjuvants share similar side effects, such as a reaction at the injection site 25 and pyrogenicity. Alum, a commonly used adjuvant for human vaccine also produces an appreciable granulomatous response at the injection site (Allison & Byars (1991) *Mol Immunol* 28:279-284). The mode of action of an incomplete Freund's adjuvant can involve non-specific as well as specific immune responses. IFA seems to work by triggering the immunocompetent cells, which are related to the inflammatory ability. IFA also acts as an antigen vehicle and a slow release or long-term antigen presentation device. Injecting a 30 patient with an IFA and antigen compound, it forms a depot of antigen at the injection site, thereby protecting the antigen from degradation. From this depot the antigen is released slowly into the system and provides a prolonged antigen presentation as well as expanded

total contact surface area and the attraction of inflammatory cells. Macrophages digest most of the incorporated materials and present the processed antigens on their surface.

The specific enhancing effect of the IFA on the antigen immunogenicity has been found to lead to increased humoral immunity (preferentially protective antibody production; 5 IgG1 in humans and IgG2a in mice) and to elicit specific cell mediated immunity (preferentially Th2 type). Specifically, human recombinant insulin B-chain in IFA results in Th2 cytokine pattern in NOD mice islets (Ramiya et al. (1996) *J Autoimmun* 9:349-356). IFA is unique among adjuvants tried for diabetes prevention in animal models. Ramiya and coworkers (*supra*) concluded that both alum and DPT as adjuvants have 'non-specific' 10 protective effects unrelated to the antigen used, while IFA is the only one with antigen specific protective effect for diabetes prevention in animals.

IFA, preferably an IFA approved for human use, e.g., Montanide (e.g., Montanide ISA51, Seppic Inc., France) or an equivalent composition, is a preferred adjuvant for use in the methods and vaccines described herein. Montanide ISA51 has shown no systemic or 15 significant local side effects in our animal studies.

Toxicology and Safety

A comprehensive toxicology/safety study on the vaccine described herein was performed. Intramuscular injection of the insulin B-chain/IFA vaccine on each of days 1, 7 and 14 to male BBDP/WOR and Sprague-Dawley rats at dose levels of 100 and 500 µg/rat, followed by a 14 day observation period had no toxicologically significant effects on clinical 20 observation, body weights, food consumption, clinical pathology (hematology, coagulation, and clinical chemistry) and organ weights. Macroscopic (all animals) and microscopic (BBDP/WOR rats in low dose, high dose and vehicle control groups) evaluation showed 25 injection site changes, including granulomatous inflammation attributable to the vehicle article.

The diabetes prone BBDP/WOR rats (the only other animal model of Type 1 diabetes apart from the NOD mice) received the insulin B-chain vaccine at a diabetes and insulitis 30 free period of their life and neither the low dose nor the high dose precipitated early insulitis or diabetes.

Serum samples from the BBDP/WOR rats (6 rats /groups) were analyzed for insulin

antibodies. There was a significant difference between the vehicle control vs. 100 µg insulin B-chain/rat and 500 µg insulin B-chain/rat doses (23.6µU/ml+3.9SE vs. 37.9µU/ml+4.5SE and 44.5µU/ml+3.3SE; significance p=0.03 and p=0.002 respectively; no significant difference between low and high dose groups in insulin antibody titers).

5 The vaccine was also analyzed for pyrogens as per the standard USP method (UPS 23<151>) and meets the requirements for absence of pyrogens.

Because we have used IFA approved for human use, this intervention strategy can be directly applied in human diabetes.

10 *Study 1*

In our animal study where we used IFA with human insulin B-chain or with human GAD65 protein, the mice were carefully observed for any adverse effect. The feeding pattern, activity, behavior as well as site of injection for local reaction were regularly monitored by the sponsor-investigator and by the research assistant and no abnormality was detected throughout of the study. The mice were vaccinated three times and all three vaccinations were well tolerated without any detectable side effects (general- feeding pattern, activity, behavior; or local- local reaction at site of injection). At the time of sacrifice, after 9 weeks the internal organs as well the vaccination sites were observed by the sponsor-investigator and no abnormality was found (any induration, swelling redness or pain).

20 *Study 2*

A second comprehensive toxicology/safety study was executed under GLP conditions in an independent FDA inspected contract laboratory (ITR Laboratories Canada Inc.). The study was designed in consultation with GAD Consulting Services and was presented to the 25 FDA prior initiation. It was deemed to be satisfactory to answer the outstanding questions of safety for this vaccine in humans. In brief, intramuscular injection of test article on each of Days 1, 7 and 14 to male BBDP/WOR and Sprague-Dawley rats at dose levels of 100 and 500 µg/rat, followed by a 14 day observation period had no toxicologically significant effects on clinical observation, body weights, food consumption, clinical pathology (hematology, coagulation, and clinical chemistry) and organ weights. Macroscopic (all animals) and 30 microscopic (BBDP/WOR rats in low dose, high dose and vehicle control groups) evaluation

showed injection site changes, including granulomatous inflammation attributable to the vehicle article.

The diabetes prone BBDP/WOR rats (the only other animal model of Type 1 diabetes apart from the NOD mice) received the IBC (insulin B-chain) vaccine at a diabetes and insulitis free period of their life and neither the low dose nor the high dose precipitated early insulitis or diabetes.

Serum samples from the BBDP/WOR rats (6 rats /groups) were analyzed for insulin antibodies. There was a significant difference between the vehicle control vs. 100 µg insulin B-chain/rat and 500 µg insulin B-chain/rat doses (23.6µU/ml+3.9SE vs. 37.9µU/ml+4.5SE and 44.5µU/ml+3.3SE; significance p=0.03 and p=0.002 respectively; no significant difference between low and high dose groups in insulin antibody titers). The IBC vaccine was prepared fresh before injecting the animals. The preparations were sampled on Day 1 and Day 14.

The vaccine was also analyzed for pyrogens as per the standard USP method (UPS 23<151>) and been reported as meeting the requirements for absence of pyrogens.

Many interventions used in NOD mice can not be considered in humans. In the results described herein, IFA approved for human use has been used, thus these intervention strategies can be directly applied in human diabetes. The IFA is safe and effective in humans. IFA is currently used in HIV and other vaccination trials (peptide-based melanoma vaccine at Univ. Virginia) approved by FDA.

Potential local side effects are similar to any commonly used adjuvant vaccinations (alum is currently used in human vaccines) and can include induration, moderate pain and low-grade fever. The injections can be given in small volume (1ml) in deep intramuscular space, thus minimizing the local side effects.

25

Administration of Vaccines

Immunogenic compositions and vaccines may be administered parenterally, by injection subcutaneously, or intramuscularly. A preferred mode of administration is intramuscularly. For example, the vaccine described herein can be given as an intramuscular injection, preferably a deep intramuscular injection, in a small volume, e.g., 1 ml. The vaccine can be administered once, or more than once. It can be given before, or shortly after

30

the onset of Type 1 diabetes mellitus.

Alternatively, the immunogenic compositions formed according to the present methods, may be formulated and delivered in a manner to evoke an immune response at a mucosal surface. Thus, the immunogenic composition may be administered by, e.g., nasal or 5 oral (intraoperative) routes. Other modes of administration include suppositories and oral formulations.

The vaccines described herein can be administered in a manner compatible with the dosage formulation, and in such amount as will be therapeutically effective, protective and immunogenic. The autoantigen in the vaccine is preferably a synthetic peptide or peptide 10 variant as opposed to a recombinant peptide or protein which is more likely to have impurities and contaminants. The quantity to be administered depends on the subject to be treated, including, for example, the capacity of the individual's immune system to synthesize antibodies, the degree of protection desired, and if needed, to produce a cell-mediated 15 immune response. Precise amounts of active ingredient required to be administered depend on the judgment of the practitioner. However, suitable dosage ranges are readily determinable by one skilled in the art and may be of the order of micrograms of the active 20 ingredient(s) per vaccination. Suitable regimes for initial administration and booster doses are also variable, but may include an initial administration followed by subsequent 25 administrations. The dosage may also depend on the route of administration and will vary according to the size of the host. For example, 2 mg of human insulin B chain solution can be administered to an adult human. The concentration of the active ingredient protein in an immunogenic composition according to the invention is in general about 1 to 95%.

A vaccine can also contain an adjuvant, e.g., an oil based adjuvant, e.g., IFA. Preferably, the vaccine contains an IFA suitable and approved for human use, e.g., 25 Montanide ISA 51 or an equivalent composition.

The vaccines are prepared under conditions suitable for human administration. Preferably, the vaccine injection is prepared as an emulsion immediately before 30 administration, under sterile conditions, by using high pressure sterile syringes as a 50/50 (w/w) emulsion of insulin B-chain/IFA.

The methods and vaccines described herein can be used to prevent the onset of an autoimmune disease, e.g., diabetes mellitus. The methods and vaccines can also be used to

arrest the autoimmune destruction of tissue, e.g., pancreatic β cells. The methods and vaccines are useful to arrest the autoimmune destruction, even at a late stage. For example, at the time of clinical onset of type 1 diabetes mellitus, significant number of insulin producing β cells are destroyed but around 15% maybe as much as 40 % are still capable of insulin production. If the autoimmune process can be arrested even in this late stage, these cells can be preserved. The β cells have some limited capacity to replicate and precursors may form new β -cells.

Autoantigen Variants

10 Variants can differ from naturally occurring protein in amino acid sequence or in ways that do not involve sequence, or both. Non-sequence modifications include *in vivo* or *in vitro* chemical derivatization of the protein. Non-sequence modifications include changes in acetylation, methylation, phosphorylation, carboxylation, or glycosylation.

15 Preferred variants include an autoantigen, e.g., preproinsulin or an immunologically active fragment thereof (e.g., insulin B-chain, A chain, C peptide), GAD65, islet tyrosine phosphatase ICA512/IA-2, ICA12, ICA69, HSP60, carboxypeptidase H, peripherin, gangliosides (e.g., GM1-2, GM3) or immunologically active fragments thereof, whose sequences differ from the wild-type sequence by one or more conservative amino acid substitutions or by one or more non-conservative amino acid substitutions, deletions, or insertions which do not abolish the immunogenic activity. In a preferred embodiment, the sequence can differ from wild-type sequence by no more than 20% of the amino acid residues. In another preferred embodiment, the sequence can differ from wild-type sequence by 1, 2, 3, 5, 10, but not more than 20 to 30 amino acid residues. Conservative substitutions typically include the substitution of one amino acid for another with similar characteristics, 20 e.g., substitutions within the following groups: valine, glycine; glycine, alanine; valine, isoleucine, leucine; aspartic acid, glutamic acid; asparagine, glutamine; serine, threonine; lysine, arginine; and phenylalanine, tyrosine. Other conservative substitutions can be taken from the table below.

TABLE I
CONSERVATIVE AMINO ACID REPLACEMENTS

For Amino Acid	Code	Replace with any of
Alanine	A	D-Ala, Gly, beta-Ala, L-Cys, D-Cys
Arginine	R	D-Arg, Lys, D-Lys, homo-Arg, D-homo-Arg, Met, Ile, D-Met, D-Ile, Orn, D-Orn
Asparagine	N	D-Asn, Asp, D-Asp, Glu, D-Glu, Gln, D-Gln
Aspartic Acid	D	D-Asp, D-Asn, Asn, Glu, D-Glu, Gln, D-Gln
Cysteine	C	D-Cys, S-Me-Cys, Met, D-Met, Thr, D-Thr
Glutamine	Q	D-Gln, Asn, D-Asn, Glu, D-Glu, Asp, D-Asp
Glutamic Acid	E	D-Glu, D-Asp, Asp, Asn, D-Asn, Gln, D-Gln
Glycine	G	Ala, D-Ala, Pro, D-Pro, β -Ala Acp
Isoleucine	I	D-Ile, Val, D-Val, Leu, D-Leu, Met, D-Met
Leucine	L	D-Leu, Val, D-Val, Leu, D-Leu, Met, D-Met
Lysine	K	D-Lys, Arg, D-Arg, homo-Arg, D-homo-Arg, Met, D-Met, Ile, D-Ile, Orn, D-Orn
Methionine	M	D-Met, S-Me-Cys, Ile, D-Ile, Leu, D-Leu, Val, D-Val
Phenylalanine	F	D-Phe, Tyr, D-Thr, L-Dopa, His, D-His, Trp, D-Trp, Trans-3,4, or 5-phenylproline, cis-3,4, or 5-phenylproline
Proline	P	D-Pro, L-I-thioazolidine-4-carboxylic acid, D-or L-1-oxazolidine-4-carboxylic acid
Serine	S	D-Ser, Thr, D-Thr, allo-Thr, Met, D-Met, Met(O), D-Met(O), L-Cys, D-Cys
Threonine	T	D-Thr, Ser, D-Ser, allo-Thr, Met, D-Met, Met(O), D-Met(O), Val, D-Val
Tyrosine	Y	D-Tyr, Phe, D-Phe, L-Dopa, His, D-His
Valine	V	D-Val, Leu, D-Leu, Ile, D-Ile, Met, D-Met

Other analogs within the invention are those with modifications which increase immunogenicity or peptide stability. Such analogs may contain, for example, one or more non-peptide bonds (which replace the peptide bonds) in the peptide sequence. Also included are analogs that include residues other than naturally occurring L-amino acids, e.g., D-amino acids or non-naturally occurring or synthetic amino acids, e.g., β or γ amino acids; and cyclic analogs.

Production of Fragments and Variants

Generation of Fragments

10 Fragments of a protein, e.g., fragments of an autoantigen described herein, can be produced in several ways, e.g., by chemical synthesis, recombinantly, or by proteolytic digestion. Chemical synthesis of immunologically active autoantigen fragments is preferred.

15 Fragments can be chemically synthesized using techniques known in the art such as conventional solid-phase peptide synthesis, e.g., Merrifield solid phase f-Moc or t-Boc chemistry. Synthetic peptides can be prepared under sterile conditions to a high level of purity, suitable for administration to humans. An autoantigen of the present invention may be arbitrarily divided into fragments of desired length with no overlap of the fragments, or divided into overlapping fragments of a desired length. These fragments can then be 20 chemically synthesized and tested for immunogenic activity as described herein below. For example, overlapping or non-overlapping fragments (e.g., fragments of between 4-100 amino acid residues, preferably between 10-60 amino acid residues, more preferably between 20-50 amino acid residues) of the 110 amino acid sequence of preproinsulin (SEQ ID NO:1) can 25 easily be made by chemical synthesis and tested for immunogenic activity by a method described herein. Fragments of any of the type 1 diabetes autoantigens described herein can be similarly made and tested.

Internal or terminal fragments of an autoantigen described herein can also be generated by removing one or more nucleotides from one end (for a terminal fragment) or both ends (for an internal fragment) of a nucleic acid which encodes the polypeptide. Expression of the mutagenized DNA produces polypeptide fragments. Digestion with "end-nibbling" endonucleases can thus generate DNA's which encode an array of fragments. 30 DNA's which encode fragments of a protein can also be generated by random shearing,

restriction digestion or a combination of the above-discussed methods, which are all known in the art.

5 Generation of Analogs: Production of Altered DNA and Peptide Sequences by Random Methods

Amino acid sequence variants of a protein can be prepared by random mutagenesis of DNA which encodes a protein or a particular domain or region of a protein, e.g., an autoantigen described herein. Useful methods include PCR mutagenesis and saturation mutagenesis. A library of random amino acid sequence variants can also be generated by the synthesis of a set of degenerate oligonucleotide sequences. (Methods for screening proteins in a library of variants are elsewhere herein.)

10 *PCR Mutagenesis*

15 In PCR mutagenesis, reduced Taq polymerase fidelity is used to introduce random mutations into a cloned fragment of DNA (Leung et al., 1989, *Technique* 1:11-15). This is a very powerful and relatively rapid method of introducing random mutations. The DNA region to be mutagenized is amplified using the polymerase chain reaction (PCR) under conditions that reduce the fidelity of DNA synthesis by Taq DNA polymerase, e.g., by using a dGTP/dATP ratio of five and adding Mn²⁺ to the PCR reaction. The pool of amplified DNA fragments are inserted into appropriate cloning vectors to provide random mutant libraries.

20 *Saturation Mutagenesis*

25 Saturation mutagenesis allows for the rapid introduction of a large number of single base substitutions into cloned DNA fragments (Mayers et al., 1985, *Science* 229:242). This technique includes generation of mutations, e.g., by chemical treatment or irradiation of single-stranded DNA *in vitro*, and synthesis of a complimentary DNA strand. The mutation frequency can be modulated by modulating the severity of the treatment, and essentially all possible base substitutions can be obtained. Because this procedure does not involve a 30 genetic selection for mutant fragments both neutral substitutions, as well as those that alter function, are obtained. The distribution of point mutations is not biased toward conserved sequence elements.

Degenerate Oligonucleotides

A library of homologs can also be generated from a set of degenerate oligonucleotide sequences. Chemical synthesis of a degenerate sequences can be carried out in an automatic 5 DNA synthesizer, and the synthetic genes then ligated into an appropriate expression vector. The synthesis of degenerate oligonucleotides is known in the art (see for example, Narang, SA (1983) *Tetrahedron* 39:3; Itakura et al. (1981) *Recombinant DNA, Proc 3rd Cleveland 10 Sympos. Macromolecules*, ed. AG Walton, Amsterdam: Elsevier pp273-289; Itakura et al. (1984) *Annu. Rev. Biochem.* 53:323; Itakura et al. (1984) *Science* 198:1056; Ike et al. (1983) *Nucleic Acid Res.* 11:477. Such techniques have been employed in the directed evolution of other proteins (see, for example, Scott et al. (1990) *Science* 249:386-390; Roberts et al. (1992) *PNAS* 89:2429-2433; Devlin et al. (1990) *Science* 249: 404-406; Cwirla et al. (1990) *PNAS* 87: 6378-6382; as well as U.S. Patents Nos. 5,223,409, 5,198,346, and 5,096,815).

15 Generation of Analogs: Production of Altered DNA and Peptide Sequences by
Directed Mutagenesis

Non-random or directed, mutagenesis techniques can be used to provide specific sequences or mutations in specific regions. These techniques can be used to create variants which include, e.g., deletions, insertions, or substitutions, of residues of the known amino acid sequence of a protein. The sites for mutation can be modified individually or in series, e.g., by (1) substituting first with conserved amino acids and then with more radical choices depending upon results achieved, (2) deleting the target residue, or (3) inserting residues of the same or a different class adjacent to the located site, or combinations of options 1-3.

25 Alanine Scanning Mutagenesis

Alanine scanning mutagenesis is a useful method for identification of certain residues or regions of the desired protein that are preferred locations or domains for mutagenesis, Cunningham and Wells (*Science* 244:1081-1085, 1989). In alanine scanning, a residue or group of target residues are identified (e.g., charged residues such as Arg, Asp, His, Lys, and 30 Glu) and replaced by a neutral or negatively charged amino acid (most preferably alanine or polyalanine). Replacement of an amino acid can affect the interaction of the amino acids with the surrounding aqueous environment in or outside the cell. Those domains

demonstrating functional sensitivity to the substitutions are then refined by introducing further or other variants at or for the sites of substitution. Thus, while the site for introducing an amino acid sequence variation is predetermined, the nature of the mutation per se need not be predetermined. For example, to optimize the performance of a mutation at a given site, 5 alanine scanning or random mutagenesis may be conducted at the target codon or region and the expressed desired protein subunit variants are screened for the optimal combination of desired activity.

Oligonucleotide-Mediated Mutagenesis

Oligonucleotide-mediated mutagenesis is a useful method for preparing substitution, deletion, and insertion variants of DNA, see, e.g., Adelman et al., (*DNA* 2:183, 1983). Briefly, the desired DNA is altered by hybridizing an oligonucleotide encoding a mutation to a DNA template, where the template is the single-stranded form of a plasmid or bacteriophage containing the unaltered or native DNA sequence of the desired protein. After hybridization, a DNA polymerase is used to synthesize an entire second complementary strand of the template that will thus incorporate the oligonucleotide primer, and will code for the selected alteration in the desired protein DNA. Generally, oligonucleotides of at least 25 nucleotides in length are used. An optimal oligonucleotide will have 12 to 15 nucleotides that are completely complementary to the template on either side of the nucleotide(s) coding for the mutation. This ensures that the oligonucleotide will hybridize properly to the single-stranded DNA template molecule. The oligonucleotides are readily synthesized using techniques known in the art such as that described by Crea et al. (*Proc. Natl. Acad. Sci. USA*, 1978, 75: 5765).

Cassette Mutagenesis

Another method for preparing variants, cassette mutagenesis, is based on the technique described by Wells et al. (*Gene*, 1985 34:315). The starting material is a plasmid (or other vector) which includes the protein subunit DNA to be mutated. The codon(s) in the protein subunit DNA to be mutated are identified. There must be a unique restriction 30 endonuclease site on each side of the identified mutation site(s). If no such restriction sites exist, they may be generated using the above-described oligonucleotide-mediated

mutagenesis method to introduce them at appropriate locations in the desired protein subunit DNA. After the restriction sites have been introduced into the plasmid, the plasmid is cut at these sites to linearize it. A double-stranded oligonucleotide encoding the sequence of the DNA between the restriction sites but containing the desired mutation(s) is synthesized using standard procedures. The two strands are synthesized separately and then hybridized together using standard techniques. This double-stranded oligonucleotide is referred to as the cassette. This cassette is designed to have 3' and 5' ends that are comparable with the ends of the linearized plasmid, such that it can be directly ligated to the plasmid. This plasmid now contains the mutated desired protein subunit DNA sequence.

10

Combinatorial Mutagenesis

Combinatorial mutagenesis can also be used to generate mutants. For example, the amino acid sequences for a group of homologs or other related proteins are aligned, preferably to promote the highest homology possible. All of the amino acids which appear at a given position of the aligned sequences can be selected to create a degenerate set of combinatorial sequences. The variegated library of variants is generated by combinatorial mutagenesis at the nucleic acid level, and is encoded by a variegated gene library. For example, a mixture of synthetic oligonucleotides can be enzymatically ligated into gene sequences such that the degenerate set of potential sequences are expressible as individual peptides, or alternatively, as a set of larger fusion proteins containing the set of degenerate sequences.

15

20

Screens for Activity

The assays described above can be followed by secondary screens to identify immunologically active variants or fragments. For example, a standard animal screening assay can be developed in which the ability of a protein variant or fragment to generate an immune response, e.g., in a rodent, can be used to identify immunologically active fragments and variants from a group of candidates isolated through one of the primary screens described above.

Therefore, methods for generating fragments and variants and testing them for activity are known in the art. Once the core sequence of interest is identified, it is routine for one of ordinary skill in the art to obtain variants and fragments.

5 Fusion Proteins

An autoantigen or variant or fragment thereof can be fused to another protein or portion thereof. For example, an autoantigen can be operably linked to another polypeptide or other moiety to enhance its stability or immunogenicity. Examples of polypeptides that can be fused with an autoantigen described herein or portions thereof include, e.g., artificial 10 T helper cell epitopes for immune stimulation of synthetic peptide immunogens (as described, e.g., in U.S. Patents 6,228,987 and 6,025,468) and other synthetic peptide carriers (See, e.g., U.S. Patent 5,736,146) and low molecular weight immune stimulants (See U.S. Patent 6,007,819).

15 EXAMPLES

20 Example 1: Preparation of insulin B-chain

Human insulin was made by standard solid-phase peptide synthesis (SPPS) procedure, described herein. The assembly strategy used in the protein synthesis was ABI (Applied Biosystem Inc.)-Fmoc/Thr. The Fmoc group protects the α -amino group of the amino acid. The peptide was assembled from the C-terminal towards the N-terminal with the α -carboxyl group of the starting amino acid attached to a solid support (resin). The resin used for assembly was polystyrene bead, an insoluble support with size of 400-1000 micron in diameter swelled after washing with NMP (N-methylpyrrolidone). The resin was 25 preloaded with the first amino acid (Thr) from the C-terminal.

30 Chain assembly

The first step in chain assembly is deprotection, or removal of the protecting group. The Fmoc protecting group is removed by 22% piperidine. Conductimetric feedback of carbamate salt formed via removal of Fmoc group with piperidine/NMP showed the coupling efficacy.

After deprotection, the next amino acid is activated and coupled to the deprotected amino end of the growing peptide and forms the peptide bond. Activation of the incoming amino acid carboxyl group was achieved by HBTU/HOBt.

Between coupling, the column was washed with methanol and NMP (N-methylpyrrolidone), which swells the resin and washes out residues.

5 The cycle is repeated until a peptide of desired length is achieved.

A wash step is performed with DCM (dichloromethane), which removes NMP from the resin, followed by thorough washing with highly volatile methanol, an easily removable solvent which evaporates and dries.

10

Cleavage from the resin and removal of side -chain protecting groups.

A cleavage mixture was prepared (0.75 g crystalline phenol + 0.25 g ethanedithiol + 0.5 ml thioanisol + 0.5 ml deionized H₂O + 10 ml trifluoroaceticacid). The dried peptide-resin was incubated in cool flask in ice bath (10 ml mixture /100-150 mg peptide-resin) for 1.5 h. Then the peptide was isolated from the reaction mixture by glass funnel filtration under high vacuum. The peptide was precipitated with cold methyl t-butyl ether (MTBE) and vacuum dried.

15 Purification under sterile condition.

This step was performed with reverse phase HPLC. Buffer A= 0.1% trifluoroaceticacid (TFA) and buffer B= 70% acetonitrile, 30% H₂O, 0.09% trifluoroaceticacid (TFA). By using a C18 column, the elution of the sample was based upon hydrophobicity (hydrphilic sample elutes earlier). The peak detection was performed by absorbance measurement of peptide bond at 214 nm and identified by mass spectrometry.

20 The desired fraction was pooled in sterile vials and lyophilized with sample taken for AAA (amino acid analysis) analytical rpHPLC and Mass Spectrometry.

25 The results of quality control tests on the B-chain produced are shown below.

30

TABLE 2: Certificate of Analysis for SPPS B-chain-final product

Parameter	Test to use	Range	Result
Appearance	Visual observation - solution	Clear	Clear
pH	pH meter	3.5-4.5	4.0
Strength	Protein concentration (Bio-Rad Assay Bio-Rad Laboratories)	3.5-4.5 mg/ml	3.82mg/ml
Immunoreactivity/potency	Human insulin RIA (Diagnostic System Laboratories Kit)	10-30 uU/mg protein/ml	80.2 uU/ml
Purity	HPLC	Area % >95%	>99%
Impurity	Total Residual Solvent (acetonitrile) gaschromatography	<500ppm (particle per million)	<20ppm (particle per million)
Identity	Mass Spectrometry	3400-3450Da	Single major peak 3428.7Da
	Aminoacid sequence Analyzer	30 aminoacid of human insulin B-chain sequence	30 aminoacid of human insulin B-chain sequence
Pyrogens	standard USP method	Meets requirement for absence of pyrogens	Meets requirement for absence of pyrogens
Sterility		Sterile	Sterile

Example 2: solubilization of insulin B-chain

The insulin B chain is a hydrophobic molecule. Preferably, the synthesized insulin B chain is solubilized and maintained in soluble form for use in the vaccine.

The insulin B-chain can be treated to increase its solubility, e.g., to counter the effect of its hydrophobicity. This can be done by acidification and/or by using 4M urea buffer and/or by reducing cysteine with DTT to avoid dimerization.

Example 3: Method of vaccine/emulsion preparation

The vaccine for human use was prepared under conditions suitable for human administration. The vaccine contains two components: an adjuvant, and insulin B-chain.

The first component is insulin B-chain, prepared and solubilized as described in Examples 1 and 2.

The second component is an IFA, e.g., Montanide ISA51 (Seppic Inc. France; Drug Master-file No:10870DMF) or an equivalent composition. This IFA has been used in our animal studies and showed no systemic or significant local side effect.

The injections were prepared fresh, immediately before administration, as an emulsion, in a lamina-flow protected hood, under sterile conditions by using high pressure sterile syringes with an 18 gauge spatial connector. 2 mg of insulin B-chain (0.5 ml) was mixed with Montanide ISA51 (0.5 ml). An equivalent composition can be used. The emulsion is a 50/50 (weight by weight) emulsion. The emulsion was given intramuscularly to an adult human subject in a 1ml volume in the thigh.

10

A number of embodiments of the invention have been described. Nevertheless, it will be understood that various modifications may be made without departing from the spirit and scope of the invention. Accordingly, other embodiments are within the scope of the following claims.

15